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(54) **Process for synthesis of polydeoxynucleotides**

(57) A process for polydeoxynucleotide synthesis from 2' - deoxynucleotide - 5' - triphosphates with a DNA polymerase enzyme that contains a free mercapto group outside the active center, in the presence of divalent cations and, if desired a template-primary complex, in buffered aqueous solution in the pH range of 6.5 to 9.5 and at a temperature of 15 to 42 °C required by the enzyme applied is carried out with the DNA polymerase enzyme covalently linked to a solid phase capable of forming bonds with mercapto groups.

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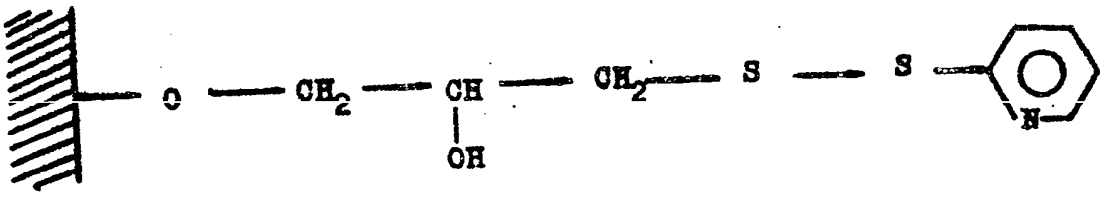
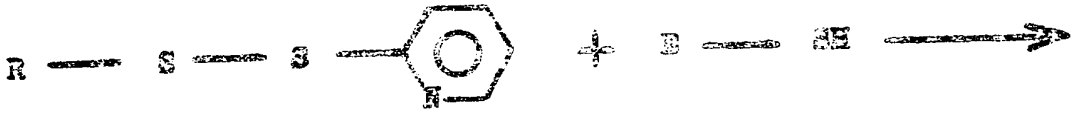
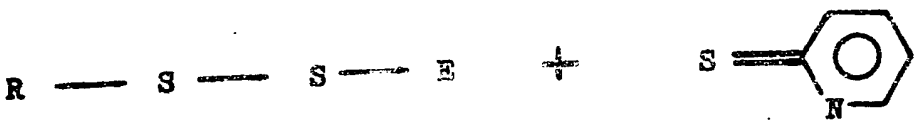
Figure 1



Reaction scheme 1





SPECIFICATION

Process for synthesis of polydeoxynucleotides

The invention relates to a process for the synthesis of polydeoxynucleotides from 2' - deoxynucleotide - 5' - triphosphates with a DNA polymerase enzyme that contains a free mercapto group outside the active center, in the presence of divalent cations and, if desired, a template-primary complex in a buffered aqueous solution in the pH range of 6.5 to 9.5 and at a temperature of 15 to 42°C required by the enzyme applied.

The template-primary complex is of the same type as the polymer molecule to be synthesized.

As is known, the enzymatic synthesis of polydeoxynucleotides is carried out in homogeneous phase reactions. Such methods are described by e.g. J. F. Burd and R. D. Wells, J. Mol. Biol, *53*, 435-459 (1970); F. J. Bollum, Procedures in Nucleic Acid Research, p. 591, G. L. Cantoni and D. R. Davies, eds., Harper and Row, New York (1966); J. Sági, A. Szabolcs, A. Szemző and L. Ötvös, Nucleic Acids Research *4*, 2767-2777 (1977). In these procedures the buffered water solution contains the substrates, the template-primary complex, enzyme and cations needed for the optimum function of the enzyme. The reaction mixture is incubated, and after completion of the reaction the enzyme is denatured (e.g. by heating up the solution), and the precipitated enzyme protein is removed from the mixture. The polydeoxynucleotide product of high molecular weight is separated from the mixture by dialysis.

Polydeoxynucleotides obtained in this way are used as DNA models for studying physical, chemical, biochemical and biological properties of nucleic acids.

A generally used DNA model is e.g. the strictly alternating double-helical copolymer of 2' - deoxyadenosine - 5' - monophosphate /dAMP/ and thymine - 5' - monophosphate /dTMP/:

. . . -dAMP-dTMP-dAMP-dTMP- . . .

which is abbreviated as poly d/A-T/.

The nucleotide sequence of a DNA strand synthesized by DNA-dependent DNA polymerase enzymes is determined by the nucleotide sequence of the template DNA applied. For preparation of poly d/A-T/ molecules, therefore, a poly d/A-T/ template molecule is required. In this way polydeoxynucleotides of different structure can be prepared in the presence of suitable template-primers, e.g. poly/DA/.poly/dT/, poly d/G-C. poly d/I-C/, poly/dG/.poly/dC/, poly/dI/.poly/dC, etc., where A = adenine, T =thymine, G = guanine, I = inosine and C = cytosine.

Certain types of DNA polymerases are capable of *de novo* synthesis of poly d/A-T, i.e., without adding exog n poly d/A-T/ to th starting reaction mixture. Such enzyme is e.g. DNA polymerase I isolated from Escherichia coli bacterium.

The disadvantage of the above known procedures for polydeoxynucleotide synthesis is that the nzyme can be used only once, and the enzyme is denatured upon its removal from the mixture in the work-up procedure.

Our aim was to dev lop a method which reduces or eliminates these disadvantages.

The basis of the invention is the recognition that this aim can be achieved if polydeoxynucleotide synthesis is carried out with a DNA polymerase enzyme fixed to a solid phase. In this way the enzyme can be removed from the reaction mixture by filtering, and can repeatedly be used.

Another basis of the invention is the recognition that DNA polymerase enzymes with free mercapto groups outside the active center can be fixed to a solid phase and then used for the above purposes. Through this mercapto /thiol/ group the enzyme can be bonded to an appropriate solid phase without loss or reduction in activity.

Based on these facts the invention offers a method for the synthesis of polydeoxynucleotides from 2' - deoxynucleotides with a DNA polymerase enzyme that contains a free mercapto group outside the active center, in the presence of divalent cations and, if desired, a template-primary complex in a buffered aqueous solution in the pH range of 6.5 to 9.5 and at a temperature of 15 to 42°C required by the enzyme applied. According to the invention a DNA polymerase enzyme fixed with a covalent bond to a solid phase that is capable of forming bonds with mercapto groups is used.

According to a preferred method of application of the process of the invention, an immobilized thiol-group-containing reagent is used as the solid phase.

For the immobilization of enzymes, solid phases capable of forming bonds with a mercapto group were found to be suitable: immobilized thiol reagents of modified agarose base, e.g. Thiopropyl-Sepharose 6B (Pharmacia Information Booklet, 1977), or Activated Thiol-Sepharose 4B (Pharmacia Information Booklet, 1974).

The structure of Thiopropyl-Sepharose 6B is shown in Figure 1. The matrix is modified agarose.Thiopropyl-Sepharose 6B contains approximately 20 μmoles of 2 - thiopyridyl groups per ml swollen gel; 1 g of freeze-dried powder reswells to give approximately 3 ml of sedimented gel. The immobilized thiol group protected and activated with a 2-thiopyridyl group can be unprotected in different ways, and can be promptly reacted with reagents that form a mixed disulphide bond. In this reaction the leaving 2-thiopyridon group has a molar extinction coefficient of 8.08×10^3 M^{-1} cm^{-1} at 343 nm according to the Pharmacia Information Booklet, 1977.

The solid phase reagent chosen is swollen in a potassium phosphate buffer (pH 7.4) and poured into a column, on top of which dissolved enzyme is applied.

In compliance with the invention an enzyme that contains a free, reactive mercapto group is used. Such enzyme is Escherichia coli DNA polym rase I (Kornberg polymerase). This is a spherical molecul of 109,000 Dalton without subunits, containing 975 amino acids. There are, among others, a lysine and an arginine molecule in the active center. The molecule contains only one disulphide bond and one thiol group. The latter can be reacted with iodoacetate or mercury salts without diminishing the activity

of the enzyme.

Similarly to DNA polymerase I isolated from E. coli, enzymes of polymerase I type isolated from other bacteria, like Bacillus subtilis, Micrococcus luteus and Mycobacterium tuberculosis, are not inhibited by sulfhydryl-blocking agents, therefor they presumably can be immobilized as the E. coli enzyme. On the other hand, enzymes of DNA polymerase types II and III isolated from the above bacteria are inhibited by sulfhydryl-blocking reagents, therefore they cannot be fixed to solid phase in the same way. Of the DNA polymerase enzymes (EC 2.7.7.7) isolated so far from mammalian sources, alpha-polymerase and gamma-polymerase are also inhibited, whereas beta-polymerase is not inhibited by sulfhydryl-blocking agents.

Enzyme layered on the top solid phase in a column is washed in with a buffer, and the solution is circulated ten times with a peristaltic pump to complete the immobilization reaction. The reaction runs according to Reaction scheme 1, where E = DNA polymerase I enzyme and

$$R = \text{Sepharose}-O-CH_2-\underset{\displaystyle OH}{\underset{|}{CH}}-CH_2-.$$

The amount of immobilized polymerase can be determined directly by measurement of the extinction coefficient of thiopyridon molecules, and indirectly by measurement of DNA synthesized in the enzyme-catalyzed reaction.

After completion of the immobilization reaction the buffer is removed, the gel is washed with a further buffer until the spectrum of the removed buffer is the same as that of the starting buffer (approx. 10 ml), then the gel is washed with an additional 40 ml of buffer at a rate of 1 ml/min. A ZEISS Specord UV VIS registrating spectrophotometer used for spectrophotometric determinations.

The polymerase enzyme fixed in the above way is then used for polydeoxynucleotide synthesis. The reaction mixture used for the synthesis contains deoxynucleotides as substrates, and template-primer in an aqueous solution.

Buffer and salt (preferably $MgCl_2$) concentrations required for optimum enzyme activity are used within the pH range of 6.5 to 9.5.

The reaction mixture (37°C) is applied to the column thermostated at 37°C, and is circulated with a peristaltic pump.

The reaction can be controlled by different methods: by radioactivity measurement of the acid-insoluble product, paper chromatography, control reaction, or by measurement of the amount of the polymer product.

Radioactivity measurement requires the presence of labelled substrates in the reaction mixture. For this purpose e.g. [^{3}H]dATP can be applied. Measurement is carried out by taking 25 μl of samples from the mixture flowing through the column with a Hamilton micropipette at different time intervals. Aliquots are pipetted onto a GF/C glass fibre filter (diameter 2.5 cm, Whatman). Filters are collected in a cold (0-5°C) solution of 5% trichloroacetic acid (TCA) and 1% sodium pyrophosphate (10 ml of solution per filter, 0-5°C, 10 minutes). After being washed twice 5% TCA, twice with 96% ethanol, and twice with diethylether, the filters are air-dried. The radioactivity of acid-insoluble polymers /above decamers/ is measured in a toluene "cocktail" in a Packard spectrometer. The control reaction can be followed in the same way.

By paper chromatography, the reaction can be followed quantitatively in the presence of radioactive substrates, and qualitatively in the presence of unlabelled substrates. 5-25 μl of samples are spotted onto DE 81 paper strips (1x15-18 cm, DEAE-Cellulose, Whatman), and chromatograms are developed with 0,15 M NH_4HCO_3: the R_f value of nucleotides is between 0.35 and 0.55, whereas polymers remain at the starting point.

To the reaction mixture removed and washed out of the gel (checked at 260 nm) a solution of 2.5 M NaCl and 0.038 M EDTA (0.65 vol) is given, and maintained at 70°C for 10 minutes. The cold mixture is then shaken three times with a mixture of chloroform-isoamylalcohol (24:1) of the same volume. The phases are separated (with centrifugation or phase separator paper, Whatman); the water phase is dialyzed against redistilled water (Bio-Fiber 50 Minibeaker, Bio-Rad Lab, Inc., 4°C). The conductivity of the water leaving the dialyzer is checked. When this decreases /at 0.8-1 liter/ to the value of the starting redistilled water (0.2-0.6 μS), an additional 0.4-0.5 liter of water is applied. The polymer is then freeze-dried, dissolved in 10^{-3}M TRIS, HCl /pH 7,4/, 10^{-4}M EDTA solution /500 μl/, and from this stock solution of polydeoxynucleotides samples are taken for determination of yield, spectra, thermal stability, and template activity. The polymer solution is stored at –25°C.

Control reaction: to a reaction mixture of 165 μl enzyme is added, and incubated at 37°C. Samples (25 μl) on GF/C filters are taken parallel with the reaction to be controlled, and the filters are elaborated as described earlier.

Storage and reuse of the immobilized enzyme can be performed as follows:

After the reaction mixture had been removed and washed out, the gel is washed with an additional 60 mm potassium phosphate buffer (100 ml), then with 20 ml of 0.02% sodium azide, and stored in this solution in the column or removed at 3-6°C. The immobilized enzyme can be reused after removal of the sodium azide solution. Only a slight decrease in activity (0-5%) can be observed. The most often reexamined and most stable preparation was the E. coli DNA polymerase I grade II enzyme: no decrease in activity could be detected after being reused six times in two months.

The main advantages of the process of the invention are as follows:

a./ DNA polymerase enzyme linked covalently to a solid phase takes part in a heterogeneous reaction in the polydeoxynucleotide synthesis and can be removed from the reaction mixture without denaturation at the end of the reaction.

b./ The immobilized enzyme can be used repeatedly.

c./ The immobilized enzyme can be us d continuously.

The process of the invention is presented in more detail by the following examples.

Example 1

a./ *Preparation of Thiopropyl-Sepharose 6B*

1 g of freeze-dried commercially prepared Thiopropyl-Sepharose 6B immobilized thiol reagent is swollen for 15 minutes at room temperature in 60 mM/l of potassium phosphate buffer (pH 7.4). The swollen gel is poured into a glass column (1.5 x 18 cm) fitted with thermostable jacket and a glass filter at the bottom, and is washed with 200 ml of the above buffer to remove preservatives.

b./ *Immobilization of DNA polymerase enzyme*

In a bubble-free buffer (approx. 0.5 ml) above the sedimented gel 100 units (20 μl) of E. coli MRE 600 DNA polymerase I, grade I enzyme (3850 units per mg protein, Boehringer-Mannheim GmbH) were pipetted with a Hamilton micropipette. (1 unit of DNA polymerase enzyme incorporates 10 nmoles of total nucleotides at 37°C during 30 minutes of incubation into an acid-insoluble product in the presence of an appropriate template-primary complex in a given buffer). The buffer containing the enzyme is allowed to flow into the gel at a rate of 0.1-0.2 ml per min., and the enzyme is washed in with an additional 10 ml buffer at room temperature. Then the whole buffer is circulated ten times with a peristaltic pump.

c./ *Synthesis of poly d/A-T/*

The column is thermostated to 37°C with a thermostate, then the following reaction mixture is poured above the gel: 60 mM/l potassium phosphate buffer (pH 7.4), 6 mM/l $MgCl_2$, 1mM [^{3}H]dATP, (specific activity: 10.9 dpm per pmol), 1mM/l dTTP, 100 μM(P)/l activated poly d/A-T/ template-primary complex in redistilled water (final volume: 10 ml). Circulation is started, the first 2-3 ml of the mixture are discarded /checked by spectrophotometry/, then the mixture is circulated with a peristaltic pump at a rate of 0.5 ml per min. The reaction is followed by radioactivity measurement of the acid-insoluble material.

After 24 hours of incubation the amount of polymer formed was 3.04 mg, based on the incorporation of [^{3}H]dAMP into the acid-insoluble product. 45% of substrates were consumed. The low molecular weight material was removed by dialysis. The amount of pure poly d/A-T/ of high molecular weight was 2.2 mg.

Example 2

150 units (100 μl) of E. coli MRE 600 DNA polym r-ase I, grade II enzyme (116 units per mg protein, Boehringer-Mannheim GmbH) were applied to an immobilized thiol reagent as described in Example 1. Synthesis was performed also as given in Example 1. The substrates used were 2 mM/l [^{3}H]dATP and 2 mM/l dTTP. The amount of polymer synthesized during 4 hours of incubation was 2.37 mg based on radioactivity measurement, and 18% of the substrates were incorporated into the polymeric material. Isolated and purified poly d/A-T/ was of low molecular weight (5000-200000 Dalton); the amount obtained was 1.78 mg.

Example 3

45 units (50 μl) of E. coli MRE 600 DNA polymerase I large fragment enzyme (Klenow enzyme, 6940 units per mg protein, Boehringer-Mannheim GmbH) were applied to the immobilized thiol reagent as described in Example 1. Synthesis was carried out according to Example 1. Substrates were 0.5 mM/l [^{3}H]dATP and 0.5 mM dTTP. Based on radioactivity measurement the amount of polymer synthesized in 24 hours of incubation was 2.74 mg, and 82% of the substrates were incorporated into polymeric material. Isolated and purified poly d/A-T/ was of high molecular weight (above 200,000 Daltons); the amount obtained was 2.12 mg.

CLAIMS

1. A process for the synthesis of polydeoxynucleotides from 2' - deoxynucleotides - 5' - triphosphates with DNA polymerase enzyme containing a free mercapto group outside the active centre, in the presence of divalent cations ands, if desired, a template-primary complex in a buffered aqueous solution in the pH range of 6.5 to 9.5 and at a temperature of 15 to 42°C required by the enzyme applied, wherein the said DNA polymerase enzyme is fixed with a covalent bond to a solid phase that is capable of forming bonds with mercapto groups.

2 A process as claimed in claim 1, in which an immobilized thiol reagent is applied as the solid phase.

3. A process as claimed in claim 1 or 2 in which said enzyme is E. coli DNA polymerase I.

4. A process as claimed in claim 1 substantially as herein described with reference to any one of the Examples.

5. Polydeoxynucleotides whenever prepared by the process claimed in any preceding claim.

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